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Exhibit J

A Rational Approach to the Design and Synthesis of a New Bradykinin B_1 Receptor Antagonist

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We have previously synthesized a potent and selective B_1 bradykinin receptor antagonist, JMV1645 (H-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH), containing a dipeptide mimetic ((3*S*)-amino-5-carbonylmethyl-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (D-BT) moiety) at the C-terminal. Analogues of this potent B_1 bradykinin receptor antagonist in which the central Pro^2-Hyp^3-Gly^4-Igl^5 tetrapeptide has been replaced by constrained N-1-substituted-1,3,8-triazaspiro[4.5]decan-4-one ring system were synthesized. Among these analogues, compound JMV1640 (**1**) was found to have an affinity of 24.10 ± 9.48 nM for the human cloned B_1 receptor. It antagonized the [des-Arg^{10}]-kallidin-induced contraction of the human umbilical vein (pA_2 = 6.1 ± 0.1). Compound **1** was devoid of agonist activity at the kinin B_1 receptor. Moreover, it did not bind to the human cloned B_2 receptor. Therefore, JMV1640 constitutes a lead compound for the rational search of nonpeptide B_1 receptor analogues based on the BK sequence.

Introduction

The kallikrein–kinin system is involved in many biological and pathophysiological processes including smooth muscle contraction, vasodilation, pain, hypotension, acute and chronic inflammation, trauma, burns, shock, and allergy.[1–5] The action of kallikreins present in plasma and tissue on kininogen generates both kallidin ([Lys]-BK, H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) and bradykinin (BK, H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH). Removal of the C-terminal Arg residue from BK and [Lys]-BK by the carboxypeptidases M and N (CPM)[6] produces a second set of biologically active peptides, [des-Arg^9]-BK and [des-Arg^{10}]-kallidin. The actions of kinins are mediated through stimulation of two receptor subtypes. B_2 receptors exhibit high affinity for BK and [Lys]-BK, while B_1 receptors have a higher affinity for the metabolites [des-Arg^9]-BK and [des-Arg^{10}]-kallidin. So far the two receptors have been cloned.[7,8] B_2 receptors are constitutively and widely expressed in central and peripheral tissues and are thought to mediate the majority of physiological actions of kinins.[9] On another hand, B_1 receptors are rarely expressed in normal tissues but are synthesized locally after tissues trauma or infection.[10] Until the past few years, although the search for B_2 receptor antagonists has been extensively undertaken, little attention has been paid to B_1 receptor antagonists. In this regard, a large number of potent peptide B_2 antagonists including the clinical evaluated second-generation HOE140[11] (H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH, Icatibant) and CP0127[12] (Bradycor) and nonpeptide antagonists[13–16] have been developed.

The growing evidence that B_1 receptors in addition to B_2 receptors play an important role in various pathophysiological processes including inflammatory pain[10,17] strengthens the need for searching for B_1 selective ligands. To develop compounds for the treatment of subacute and chronic inflammatory disorders, peptides with combined B_1 and B_2 antagonist activity[18,19] or selective potent B_1 receptor antagonists were developed.[20] More recently, Ferrari et al.[21] reported the first nonpeptide B_1 receptor antagonists based on *N*-(arylsulfonyl)amino acid derivatives. Chakravarty et al.[22] and Mavunkel et al.[23] described a series of B_2 receptor antagonist analogues of HOE140 in which the central Pro^2-Hyp^3-Gly^4-Thi^5 tetrapeptide was replaced by alkyl-, cycloalkyl-, aryl-, and aralkyl-substituted 1,3,8-triazaspiro[4.5]decan-4-one-3-acetic acid moieties.[24,25] They proposed the 1,3,8-triazaspiro[4.5]decan-4-one ring system substituted with an alkyl or aryl group at position 1 as a rigid tetrapeptide Pro^2-Pro^3-Gly^4-Phe^5 mimetic. B_1 receptor agonists were also synthesized by replacing the tetrapeptide Pro-Pro-Gly-Phe by alkyl spacers in [des-Arg^9]-bradykinin analogues.[26] More recently, Gallopini et al.[27] applied the same approach to design B_1 receptor antagonists using nonrigid or semirigid tetrapeptide mimetics. They have reported analogues of [des-Arg^{10}]-HOE140 and B9858 (H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-OH) in which the central Pro-Hyp-Gly-Xaa was replaced by linear spacers of variable length. The most potent compound, MEN 11575 (H-D-Arg-Arg-NH-$(CH_2)_{10}$-CO-Ser-D-Tic-Oic-OH), characterized by a linear and flexible spacer showed B_1 receptor antagonist activity giving a pA_2 of 7.1 on the rat ileum longitudinal smooth muscle.

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# UMR C9955 CNRS-Inserm U414.

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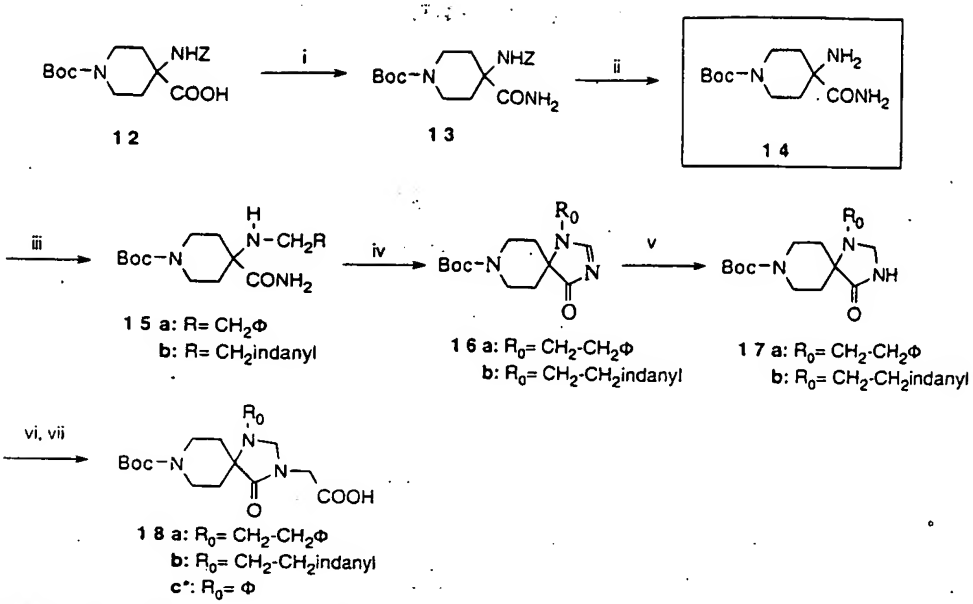
Scheme 1[a]



[a] Reagents: (i) NMM, IBCF, DME, −15 °C then NH_4OH; (ii) H_2, Pd/C, EtOH (95%); (iii) RCHO, $NaBH_3CN$, MeOH, AcOH (1%); (iv) $(EtO)_3CH$, H^+, toluene; (v) $NaBH_4$, EtOH; (vi) $BrCH_2COOEt$, NaH, DMF, 0 °C; (vii) 1 N NaOH, EtOH. *Compound **18c** is commercially available.

The role of the tetrapeptide Pro-Pro-Gly-Phe is supposed to anchor the bradykinin analogue to the membrane environment and maintain the Lys^0 and Phe^8 at the correct distance and in the optimal topological orientation for interaction with the receptor.[28]

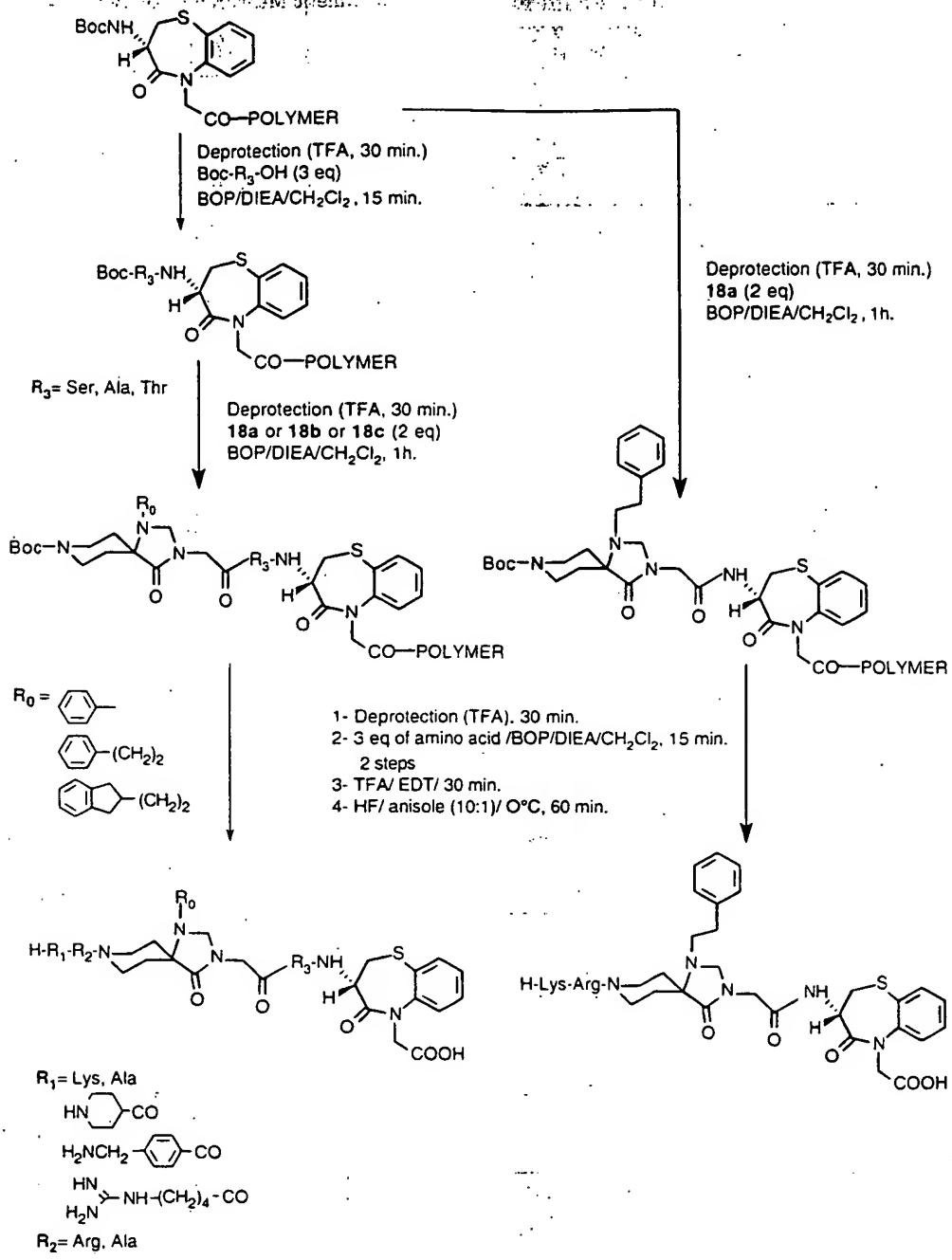
We recently described a series of B_2 receptor agonists in which the dipeptide Pro-Phe of BK or D-Tic-Oic of HOE140 was replaced by a benzothiazepinone (D-BT) moiety.[29,30] We also reported the synthesis of B_1 receptor antagonists based on the substitution of the dipeptide D-Igl-Oic of the potent B_1 receptor antagonist B9858 (H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-OH)[20] by a benzothiazepinone (D-BT) moiety.[31,32] Among the most potent compounds, JMV1645 (H-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH) had a K_i value of 0.023 ± 0.001 nM on the human cloned B_1 receptor and a pA_2 of 8 on the human umbilical vein. Therefore, we focused our efforts on the synthesis of constrained peptidomimetics in order to approach a rational design of nonpeptide compounds. In this respect, using JMV1645 as a model pseudopeptide, we replaced the tetrapeptide Pro^2-Hyp^3-Gly^4-Igl^5 by molecular scaffold analogues to those previously described.[22,23] Replacement of Pro^2-Hyp^3-Gly^4-Igl^5 by linear alkyl spacers leading to compounds of general structure H-Lys-Arg-NH-$(CH_2)_n$-CO-Ser-D-BT-OH resulted in compounds with low affinity, for both the B_1 and B_2 receptors. However, substitution by the *N*-1-phenethyl-1,3,8-triazaspiro[4.5]decan-4-one ring system yielded compound **1** (JMV1640) which displayed high affinity and selectivity for the human cloned B_1 receptor (Scheme 3). A structure−activity relationship of this lead compound is described.

Chemistry

The constrained dipeptide mimetic Boc-D-BT-OH was prepared as previously describ d.[29] 1-(2-Phenylethyl)-8-Boc-1,3,8-triazaspiro[4.5]decan-4-one-3-acetic acid (**18a**) and 1-[2-(2-indanyl)ethyl]-8-Boc-1,3,8-triazaspiro[4.5]-decan-4-one-3-acetic acid (**18b**) were prepared according to Scheme 1. N^{α}-Z-N^{γ}-Boc-protected 4-aminopiperidine-4-carboxylic acid (Z-Pip(Boc)-OH, **12**) was prepared according to Wysong et al.[33] It reacted with isobutyl chloroformate in the presence of *N*-methylmorpholine and aqueous ammonia to yield the corresponding amide **13**. Hydrogenolysis over palladium on charcoal of **13** removed the benzyloxycarbonyl protecting group to produce **14**. Reductive amination of **14** with the appropriate aldehyde in the presence of sodium cyanoborohydride yielded the amino amides **15a,b**. 2-Indanylacetaldehyde was obtained from 2-indanylacetic acid via reduction of its Weinreb amide.[34,35] The use of different aldehydes allowed the introduction of diversity onto the spiro compounds. Starting from amino amides **15a,b**, 1,3,8-triazaspiro[4.5]decan-4-ones **18a,b** were prepared as described in the literature.[23]Amines **15a,b** were treated with triethyl orthoformate to yield the spirocyclic compounds **16a,b**. Reduction of **16a,b** by $NaBH_4$ in EtOH led to compounds **17a,b**. Alkylation of the amide fonction with ethyl bromoacetate followed by hydrolysis of the ethyl ester yielded the various Boc-3-carboxymethyl-1-substituted-1,3,8-triazaspiro[4.5]decan-4-ones **18a,b**.

Bradykinin analogues **1**−**11** (Table 1) incorporating spirocyclic scaffolds **18a**−**c** were prepared using standard solid-phase coupling procedures. The synthetic route used for their preparation is described in Scheme 2. All D-BT-containing peptides were synthesized on a chloromethylated resin by the solid-phase method with Boc-D-BT-OH bound to the resin via its cesium salt.[36] N^{α}-*tert*-Butyloxycarbonyl (Boc) group was used as temporary protection of the N-terminal amino groups, and tosyl, benzyloxycarbonyl (Z), and benzyl groups were used for side-chain protection. Coupling of protected amino acids was carried out with BOP reagent.[37] After deprotection of the Boc group by TFA in the presence of ethanedithiol as scavenger, BK analogues were deprotected and cleaved from the resin using standard liquid HF procedure in the presence of anisole. Com-

Scheme 2. Synthesis of Compounds 1–11



pounds **1**–**11** were purified by preparative reverse-phase HPLC on a C_{18} column, and their purity was checked by analytical HPLC and capillary electrophoresis. Analytical data are reported in Table 2. Compounds were characterized by mass spectrometry and ^1H NMR spectroscopy.

Results

NMR Characterization of Compound 1. The chemical structure of the peptidomimetic **1** was fully checked by NMR spectroscopy. For this purpose DQF-COSY, TOCSY, NOESY, ROESY, HSQC, and HSQC-TOCSY experiments were recorded. The assignments of ^1H and ^{13}C resonances are given in Table 3.

Surprisingly, in the 1D spectrum all the amide signals were observed as two doublets of approximately th

Scheme 3

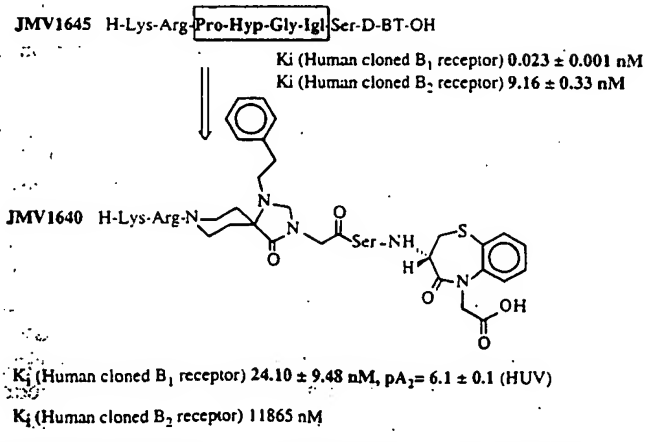
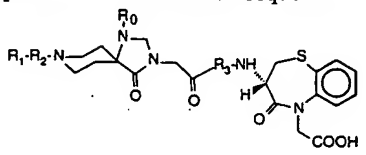


Table 1. Pseudopeptides 1–11 of General Sequence



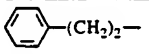
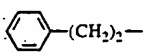
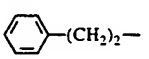
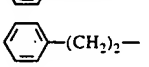
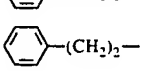
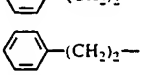
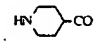
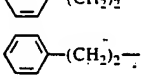
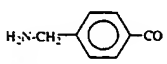
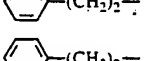
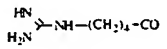
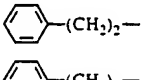
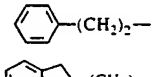
Peptides	R_1	R_2	R_0	R_3
1 (JMV1640)	Lys	Arg		Ser
2	Ala	Arg		Ser
3	Lys	Ala		Ser
4	Lys	Arg		Ala
5	Lys	Arg		Thr
6	Lys	Arg		–
7		Arg		Ser
8		Arg		Ser
9		Arg		Ser
10	Lys	Arg		Ser
11	Lys	Arg		Ser

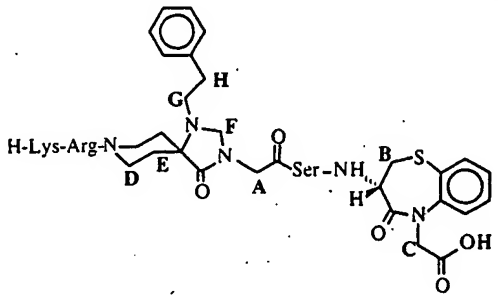
Table 2. Analytical Characteristics of B_1 Receptor Antagonist Analogues

compd	HPLC[a] t_R, min	CZE[b] t_R, min	ESI-MS[c] (M + H$^+$)
1	28.3	5.25	923
2	18.3	3.18	866
3	18.4	3.27	838
4	18.5	3.32	907
5	27.3	7.41	937
6	19.1	3.36	836
7	14.6	4.89	906
8	16.7	5.90	928
9	16.9	5.06	936
10	19.9	5.46	963
11	15.5	6.58	894

[a] HPLC was performed with a NewGold Beckman instrument on a Delta-Pak C_{18} column (5 μm, 100 Å, 3.9 × 150 mm) at a flow rate of 1 mL/min of a binary eluent system A/B (A: H_2O, TFA 0.1%; B: CH_3CN, TFA 0.1%) with UV detection at 214 nm. Used gradient was: 0% B 50 min→ 100% B. Purity of all compounds >98%. [b] Capillary electrophoresis was performed on a PACE 5000 Beckman instrument, using an uncoated fused silica capillary (75 μm × 50 cm × 800 μm aperture), pressure injection and run conditions of 15 min, 20 °C, 30 kV, 25 mM phosphate buffer, pH 2.6. Purity of all compounds >98%. [c] ESI mass spectrometry was run on a Ltd. instrument.

same intensity and close chemical shift instead of a single doublet as usual (Figure 1). An increase of the temperature from 27 to 67 °C reduced their chemical shift differences (Figure 1) suggesting the presence of two conformations in slow exchange on the proton chemical shift time scale. The presence of these two conformations could be explained either from the cis/trans conformation for one of the three N-substituted amide bonds or by the inversion of the piperidine ring as observed for cyclohexane.[38] Interestingly, carbon signals of the piperidine ring display two sets of chemical shifts in the 37–41 ppm range for each carbon linked to the nitrogen (Table 3). These two sets correspond to the chemical shifts of the two chair conformations of the ring, the proton spectrum being more complex since axial and equatorial protons have different chemical shifts. The ROESY experiment recorded at 47 °C showed exchange cross-peaks between protons of these two sets of carbon signals. These results suggest that inversion of the piperidine ring would be responsible for the observed conformational equilibrium rather than cis/trans isomerization of one N-substituted amide bond. Such an equilibrium in which one conformation was favored (60/40) was also observed in water (data not shown). It was also observed for the other compounds of this family. Therefore, this conformational mobility

Table 3. Proton and Carbon Chemical Shifs (ppm) of the Peptidomimetic Measured in DMSO-d_6 (27 °C) of Compound 1



residue	group	proton (ppm)	carbon[a] (ppm)	temp coeff (10^{-3} ppm/°C)
Lys	H3N+	8.14		
	CαH	3.78	51.3	
	CβH2	1.67	30.1	
	CγH2	1.30	20.4	
	CδH2	1.49	25.9	
	CεH2	2.73 and 2.71[b]	38.0	
	H3N+	7.74 and 7.70[b]		
Arg	HN	8.67 and 8.65[b]		−3.4 and −3.1
	CαH	4.73	48.0	
	CβH2	1.63–1.50	27.9	
	CγH2	1.50	24.2	
	CδH2	3.09 and 3.08[b]	40.0	
	εNH	7.74 and 7.67[b]		
Pip[c]	CH2 D	4.11–3.25	37.7	
	CH2 D′ and	3.96–3.29	37.2	
	CH2 D	3.80–3.67	41.0	
	CH2 D′	3.67	40.8	
	CH2 E	1.60–1.44[b]	27.9	
	CH2 E′ and	1.59	27.9	
	CH2 E′	1.44	27.9	
A	CH2	3.96	42.4	
Ser	HN	8.19 and 8.17[b]		−4.7 and −4.6
	CαH	4.33	54.4	
	CβH2	3.50	61.4	
DBT	HN	8.41 and 8.39[b]		−6.8 and −6.8
	CαH	4.50	48.5	
	CβH2	3.44–3.00	36.8	
	CH	(1d) 7.67	134.8	
	CH	(2t) 7.34	127.1	
	CH	(3t) 7.55	130.3	
	CH	(4d) 7.47	123.7	
C	CH2	4.61–4.27	50.3	
F	CH2	4.21	65.4	
phenyl	C2–6H	7.25	127.6	
	C3–5H	7.25	128.2	
	C4H	7.19	125.5	
H	CH2	2.67	33.5	
G	CH2	2.72–2.68	47.2	

[a] Chemical shifts were measured on HSQC; therefore, chemical shifts of carbons linked to protons are reported. [b] Chemical shifts measured for the two conformers. [c] Piperidyl moiety.

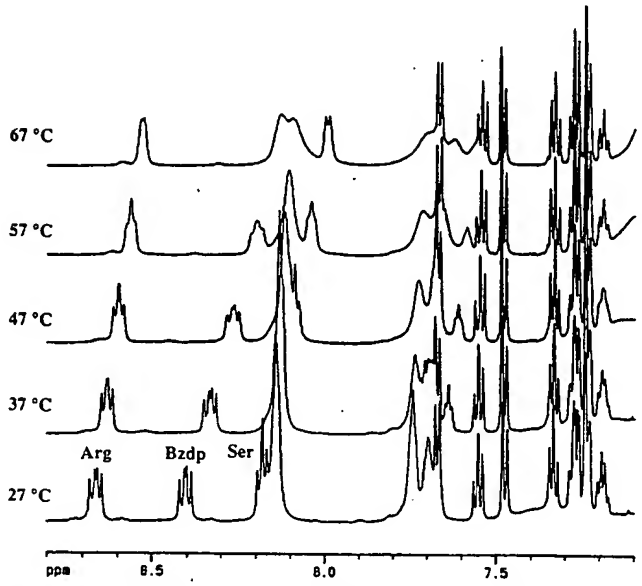


Figure 1. Spectra of the peptidomimetic **1** recorded as a function of temperature from 27 to 67 °C (DMSO-d_6, 600 MHz). At 27 °C, all amide signals are duplicated due to the presence of two conformers in slow exchange on the proton chemical shift time scale. An increase of the temperature speeds up the exchange and amide signals are progressively converted to one doublet. Amide signals are labeled.

observed by NMR cannot be used to explain the differences in affinity for the B_1 receptor.

Pharmacology. Compounds **1**–**11** were tested for their ability to compete with the specific binding of either [^{3}H][des-Arg^{10}-Leu^9]-kallidin or [^{3}H]BK to membrane preparations from 293 cells expressing the human cloned B_1 receptor[39] and CHO cells expressing the human cloned B_2 receptors,[40] respectively. Compounds having a potent binding affinity at the B_1 receptor were also tested for their ability to antagonize [des-Arg^{10}]-kallidin-induced contraction of the human umbilical vein.

Discussion

Substitution of the Pro^2-Hyp^3-Gly^4-Igl^5 tetrapeptide in JMV1645 (H-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH) by a 1-(2-phenylethyl)-1,3,8-triazaspiro[4.5]decan-4-one ring moiety led to compound **1** which was able to bind the human cloned B_1 receptor giving a K_i of 24.10 ± 9.48 nM (Scheme 3). However, this compound did not show any affinity for the B_2 receptor. It selectively and competitively inhibited contraction of the human umbilical vein induced by the potent B_1 receptor agonist [des-Arg^{10}]-kallidin giving a calculated pA_2 of 6.1 ± 0.1. These results prompted us to investigate the structure–activity relationship (SAR) of compound **1**. We first studied the role of the three remaining amino acids of the molecule. To check the importance of the basic groups at the N-terminus, we prepared analogues of **1** in which lysine and arginine residues were alternatively substituted by an alanine to yield respectively **2** and **3**. These two compounds presented no significant affinity at the B_1 receptor at a concentration up to 10 μM. Similarly, replacement of the N-terminal lysine by isonipecotyl, 4-aminomethylbenzoyl, and 5-guanidinopentanoyl moieties (e.g. compounds **7**–**9**) resulted in inactive compounds. It has been reported[20] that the presence of a basic N-terminal residue was necessary for high-affinity binding at the B_1 receptor. In our case, any modification affecting one of the two basic residues resulted in analogues with no affinity for the B_1 receptor.

We have also investigated the substitution of Ser by Thr or Ala in compound **1** as well as its deletion. Again and unexpectedly, none of the three resulting compounds, e.g. **4**–**6**, retained binding affinity for the B_1 receptor.

To further investigate the N-1 substitution in the spirocyclic moiety, we carried out the synthesis of various N-1-substituted spirocyclic scaffolds. It has been described in the literature that the introduction of α-(2-indanyl)glycine in BK antagonists sequences[19] and phenyl substituent[23,41] in BK-containing spirocyclic structures yielded analogues of remarkable affinity. Taking into account these results we introduced indanethyl and phenyl substituents at the N-1 position of the mimetic. The resulting compounds **10** and **11** did not bind to B_1 receptors at a concentration up to 10^{-5} M. None of the compounds **1**–**11** bound to the B_2 receptor.

The SAR of **1** was rather unexpected. Any modification that was performed resulted in an almost complete loss of affinity to the B_1 receptor. These data support the idea that the presence of D-BT and a spiro ring system may produce a rather highly contrained compound difficult to optimize by classical modifications. However, one can expect that defined and restricted modification will result in highly active compounds. In this respect, JMV1640 which is active and selective at the bradykinin B_1 receptor remains an interesting lead compound for the rational design of potent nonpeptide B_1 receptor antagonists.

Experimental Section

Compounds **1**–**11** were prepared by solid-phase synthesis using a Boc strategy. The starting chloromethylated Merrifield resin was purchased from Pierce; Boc-amino acids were obtained from Bachem. The (3*S*)-[*tert*-butyloxycarbonylamino]-5-carboxymethyl-2,3-dihydro-1,5-benzothiazepin-4(*5H*)-one (Boc-D-BT-OH) and 1,3,8-triazaspiro[4.5]decan-4-one-3-acetic acid moieties were synthesized in our laboratory. The spirocyclic derivative **18c** was purchased from Neosystem Laboratoire (Strasbourg, France). The following protected amino acids were used: Boc-Lys(Z)-OH, Boc-Arg(Tos)-OH, Boc-Ser(Bzl)-OH. Boc-D-BT-OH was coupled to the chloromethylated resin (1–2 mmol/g) according to the Gisin method[42] to afford a functionalized resin Boc-D-BT-O-Merrifield with a fonctionnality in the range of 0.98 mmol/g.

Final compounds were cleaved from the resin by HF in the presence of anisole and crude products were purified by reverse-phase HPLC on a Waters Delta-Prep 4000 chromatography equipped with a Waters 486 UV detector with detection at 214 nm, using a Delta-Pak C_{18} column (40 × 100 mm, 15 μm, 100 Å) at a flow rate of 50 mL/min of a binary eluent system of A/B (A: H_2O, TFA 0.1%; B: CH_3CN, TFA 0.1%). Melting points were taken on a Büchi apparatus in open capillary tubes. Compounds were identified by ^{1}H NMR at 600 MHz. As an exemple, NMR values of the potent compound **1** are reported in Table 3.

General Procedure for the Preparation of Pseudopeptides 1–11. Boc-D-BT-O-Merrifield (255 mg; 0.25 mmol) was used and the following amino acids were coupled in DCM in the presence of BOP as coupling reagent and DIEA, to the growing peptide chain in stepwise fashion: 3 equiv of Boc-Ser(Bzl)-OH (or Boc-Thr(Bzl)-OH or Boc-Ala-OH), 2 equiv of acids **18a–c**, 3 equiv of Boc-Arg(Tos)-OH (or Boc-Ala-OH), and 3 equiv of Boc-Lys(Z)-OH (or Boc-Ala-OH or Boc-isonipecotic

acid or Boc-(4-aminomethyl)benzoic acid or 5-(*N,N'*-bis-Boc-guanidino)pentanoic acid). Reaction times for complete couplings were 15 min for Boc-Ser(Bzl)-OH, Boc-Thr(Bzl)-OH, Boc-Ala-OH, Boc-Arg(Tos)-OH, Boc-Lys(Z)-OH, Boc-Ala-OH, Boc-isonipecotic acid, 4-(Boc-aminomethyl)benzoic acid and 5-(*N,N'*-bis-Boc-guanidino)pentanoic acid and 1 h for acids **18a–c**. Completion of the reaction was checked by the Kaiser test. N-α-Boc deblocking was achieved with a mixture of TFA/DCM/ethanedithiol (40/60/2). Washings of the substituted resin were performed with isopropyl alcohol and DCM. After coupling of the last amino acid, the Boc protecting group was removed by TFA/DCM/ethanedithiol (40/60/2). The total deprotection and the cleavage from the resin were performed with HF. The peptidyl resin was placed in a Teflon reactor containing anisole (1 mL/g of resin). After distillation of HF (10 mL/g of resin) into the reactor, the mixture was stirred 1 h at 0 °C. HF was removed by distillation. The expected pseudopeptide was precipitated by addition of ether, washed with ether and finally purified by reverse-phase HPLC.

1-Boc-4-[Z-amino]piperidine-4-carboxamide, 13. To a cold (−15 °C) solution of N^{α}-Z-N^{γ}-Boc-protected 4-aminopiperidine-4-carboxylic acid (Z-Pip(Boc)-OH, **12**) (19 g, 50 mmol) in DME (100 mL) were successively added NMM (5.6 mL, 50 mmol) and IBCF (6.6 mL, 50 mmol). After 5 min stirring, concentrated NH_4OH (10 mL) was added and the mixture was stirred for 1 h at room temperature. Ethyl acetate (200 mL) was then added and the solution washed with water, brine, dried over magnesium sulfate and concentrated under reduced pressure. The residue was crystallized from ether to afford **13** (16 g, 85%) as a white powder: mp 178–182 °C; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.48 (9 H, s), 2.09 (4 H, m), 3.12 (2 H, m), 3.86 (2 H, m), 5.03 (1 H, s), 5.13 (2 H, s), 5.37 (1 H, s), 6.63 (1 H, s), 7.38 (5 H, m); MS (ESI) *m/z* 378 (M + H); HPLC t_R = 20.06.

1-Boc-4-aminopiperidine-4-carboxamide, 14. Compound **13** (15 g, 39.8 mmol) was hydrogenated overnight at room temperature in 95% EtOH containing concentrated hydrochloric acid (3.6 mL, 39.8 mmol) in the presence of a 10% Pd/C catalyst. The catalyst was then removed by filtration and the filtrate concentrated in vacuo to leave a residue that solidified upon trituration in ether. It was collected, washed with ether and dried in vacuo over KOH pellets: yield 11.1 g (100%); mp 188–192 °C; ^{1}H NMR (DMSO, 250 MHz) δ 1.42 (9 H, s), 1.74 (2 H, m), 2.09 (2 H, m), 3.22 (2 H, m), 3.76 (2 H, m), 7.76 (1 H, s), 7.94 (1 H, s), 8.43 (2 H, s); MS (ESI) *m/z* 244 (M + H); HPLC t_R = 8.4.

1-Boc-4-[(2-phenylethyl)amino]piperidine-4-carboxamide, 15a. The partially deprotected amino amide **14** (5 g, 17.9 mmol) and phenylacetaldehyde (2.5 mL, 17.9 mmol) were dissolved in methanol (50 mL) with 1% acetic acid. The mixture was stirred for 30 min at room temperature. Then, a solution of sodium cyanoborohydride (2.2 g, 35.8 mmol) in MeOH was added dropwise over 30 min. After an additional 1 h the solvent was removed under reduced pressure and the residue treated with sodium bicarbonate solution (100 mL). It was extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with water (1 × 50 mL), dried over sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography, with ethyl acetate as eluent, to yield a pure compound that gave a white powder by trituration with ether: 3.85 g (62%); mp 55–57 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.47 (9 H, s), 1.56 (2 H, m), 2.02 (2 H, m), 2.79 (4 H, s), 3.03 (2 H, m), 3.77 (2 H, m), 5.18 (1 H, br s), 6.75 (1 H, br s), 7.23–7.36 (5H, m); MS (ESI) *m/z* 348 (M + H); HPLC t_R = 15.13.

1-Boc-4-[(2-indanylethyl)amino]piperidine-4-carboxamide, 15b. Indanylacetaldehyde (1.6 g, 10 mmol) obtained from the *N,O*-dimethylhydroxamate derivative of 2-indanylacetic acid[35] was dissolved in a mixture of methanol/acetic acid, 99:1 (30 mL), containing N^{γ}-Boc-protected 4-aminopiperidine-4-carboxamide (H-Pip(Boc)-NH_2) (2.79 g, 10 mmol). The mixture was stirred for 30 min at room temperature. Then, a solution of sodium cyanoborohydride (1.24 g, 20 mmol) in MeOH was added dropwise over 30 min. After an additional 1 h the solvent was removed under reduced pressure and the residue treated with sodium bicarbonate solution (50 mL). It was extracted with ethyl acetate (3 × 30 mL). The combined extracts were washed with water (1 × 30 mL), dried over sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography, with ethyl acetate as eluent, to yield a pure compound that gave a white powder by trituration with ether: 2.5 g (65%); mp 50–55 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (9 H, s), 1.63 (2 H, m), 1.72 (2 H, m), 1.88 (1H, m), 2.05 (2 H, m), 2.56–2.66 (4 H, m), 3.09 (2 H, m), 3.21 (2 H, m), 3.81 (2 H, m), 5.76 (1 H, br s), 7.06 (1 H, br s), 7.15–7.24 (4 H, m); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 28.8, 32.6, 32.6, 37.3, 39.5, 40.4, 44.5, 59.6, 80.0, 126.9, 129.0, 129.2, 139.9, 155.1, 179.1; MS (ESI) *m/z* 388 (M + H); HPLC t_R = 18.62.

1-(2-Phenylethyl)-8-Boc-1,3,8-triazaspiro[4.5]dec-2-en-4-one, 16a. A mixture of **15a** (3.37 g, 10 mmol), triethyl orthoformate (7 mL, excess), and acetic acid (1.7 mL) in toluene was refuxed for 15 h. The mixture was cooled at room temperature and concentrated in vacuo to a volume of 10 mL. Then, water (40 mL) and $CHCl_3$ (40 mL) were added followed by a 2 N aqueous solution of sodium hydroxyde. The aqueous layer was extracted with $CHCl_3$ (3 × 30 mL). The combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford 3 g (85%) of the title compound as a white powder: mp 55–60 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.51 (9 H, s), 1.55 (2 H, m), 1.83 (2 H, m), 2.99 (2H, t, J = 7.08 Hz), 3.58 (2H, t, J = 7.08 Hz), 3.65 (2 H, m), 3.71 (2 H, m), 7.18 (2H, d, J = 8.2 Hz), 7.3–7.4 (3 H, m), 7.86 (1H, s); MS (ESI) *m/z* 358 (M + H); HPLC t_R = 19.61.

1-(2-Indanylethyl)-8-Boc-1,3,8-triazaspiro[4.5]dec-2-en-4-one, 16b. Obtained as described for compound **16a** from 1.45 g of **15b**: yield 1.2 g (80%); mp 70–72 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.53 (9 H, s), 1.79 (2 H, m), 1.96–2.09 (4 H, m), 2.53 (1 H, m), 2.69 (2 H, dd, J_1 = 7.74 Hz, J_2 = 15.34 Hz), 3.16 (2 H, dd, J_1 = 7.76 Hz, J_2 = 15.14 Hz), 3.51–3.62 (4 H, m), 4.16 (2 H, m), 7.18–7.25 (4 H, m), 7.30 (1 H, s); MS (ESI) *m/z* 398 (M + H); HPLC t_R = 23.07.

1-(2-Phenylethyl)-8-Boc-1,3,8-triazaspiro[4.5]decan-4-one, 17a. To a solution of compound **16a** (2 g, 5 mmol) in ethanol (100 mL) was added sodium borohydride (250 mg, 2 equiv). The mixture was heated at 80 °C for 4 h. The solvent was then removed in vacuo and the residue was dissolved in 100 mL of ethyl acetate. This solution was washed with water and brine, dried over $MgSO_4$, filtered and concentrated in vacuo to afford the tittle compound as a white solid: yield 1.9 g (96%); mp 140–144 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.48 (9 H, s), 1.66 (4 H, m), 2.79 (4 H, m), 3.50 (2 H, m), 3.96 (2 H, m), 4.22 (2 H, s), 6.25 (1 H, br s), 7.21–7.35 (5 H, m); MS (ESI) *m/z* 360 (M + H); HPLC t_R = 18.59.

1-(2-Indanylethyl)-8-Boc-1,3,8-triazaspiro[4.5]decan-4-one, 17b. Obtained as described for compound **17a** from 1.1 g of **16b**: yield 1 g (90%); mp 138–142 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.48 (9 H, s), 1.78–1.95 (7 H, m), 2.64–2.84 (4 H, m), 3.21 (2 H, dd, J_1 = 7.53 Hz, J_2 = 15.10 Hz), 3.62–3.69 (2 H, m), 4.14–4.20 (2 H, m), 4.40 (2 H, m), 6.20 (1 H, br s), 7.26–7.34 (4 H, m); MS (ESI) *m/z* 400 (M + H); HPLC t_R = 21.7.

1-(2-Phenylethyl)-8-Boc-1,3,8-triazaspiro[4.5]decan-4-one-3-acetic Acid, 18a. Compound **17a** (1.5 g, 4.2 mmol) was dissolved in 20 mL of anhydrous DMF and cooled at 0 °C. Sodium hydride (116 mg, 5 mmol) was added all at once, and the reaction mixture was stirred for 15 min. Then ethyl bromoacetate was added (468 mL, 4.2 mmol) and the solution left at room temperature for 5 h. The solution was concentrated to a small volume and ethyl acetate (50 mL) was added followed by water (10 mL). After 1 h at room temperature, 40 mL of water was added. The aqueous phase was acidified to pH 4 with 1 N HCl and extracted with ethyl acetate (3 × 30 mL). The combined organics extracts were washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford 1.6 g (91%) of a white solid: mp 78–82 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.48 (9 H, s), 1.63–1.76 (4 H, m), 2.80 (4 H, m), 3.43–3.52 (2 H, m), 3.89–4.02 (2

H, m), 4.1 (2 H, m), 4.33 (2 H, s), 7.21–7.35 (5 H, m); MS (ESI) *m/z* 418 (M + H); HPLC t_R = 20.23.

1-(2-Indanylethyl)-8-Boc-1,3,8-triazaspiro[4.5]decan-4-one-3-acetic Acid, 18b. Obtained as described for compound **18a** from 900 mg of **17b**: yield 880 mg (85%); mp 80–82 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (9 H, s), 1.68–1.82 (7 H, m), 2.51–2.69 (4 H, m), 3.09 (2 H, dd, J_1 = 7.74 Hz, J_2 = 15.17 Hz), 3.46–3.60 (2 H, m), 4.05 (4 H, m), 4.29 (2 H, s), 7.19 (4 H, m); MS (ESI) *m/z* 458 (M + H); HPLC t_R = 22.42.

NMR Spectroscopy. The sample was solubilized in DMSO-d_6 and all NMR experiments were recorded at 27 °C on a Bruker AMX spectrometer, operating at 600 MHz for ^{1}H nucleus and 150 MHz for ^{13}C nucleus. Chemical shifts are quoted relative to the DMSO-d_6 resonance fixed at 2.5 ppm for the proton and 39.5 ppm for the carbon. DQF-COSY spectra (Rance et al.)[43] were collected into a 800 × 1024 data matrix with 32 scans per t_1 value and TOCSY (Rance)[44] spectra were collected with a mixing time of 80 ms into a 512 × 1024 data matrix with 16 scans per t_1 value. NOESY[45] spectra were acquired in the phase-sensitive mode using time-proportional phase incrementation with a mixing time of 250 ms. A ROESY experiment[46] with a mixing time of 200 ms was recorded at 47 °C to identify signals involved in the conformational exchange. HSQC[47] experiment was recorded with a delay of 3.5 ms ($^1J_{CH}$ = 143 Hz) and HSQC-TOCSY experiment with a mixing time for proton–proton transfer of 80 ms (400 × 1024) with 32 scans per t_1 value to identify the one-bond carbon–proton and the network of proton–proton connectivities, respectively.

All data were processed with the UXNMR software. For DQF-COSY and TOCSY data, one zero filling and a $\pi/4$ phase-shifted sine bell window function were applied in both dimensions before Fourier transform. For HSQC and HSQC-TOCSY data, a zero filling and a $\pi/2$ phase-shifted sine bell window function were applied in F2 dimension prior to processing.

Pharmacology. Materials. HOE140 (D-Arg^0-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin), [Leu^8,des-Arg^9]-BK, [Leu^9,des-Arg^{10}]-KD and [des-Arg^{10}]-HOE140 were synthesized in our laboratory. MERGETPA (DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid) was obtained from Calbiochem (La Jolla, CA). All molecular biology and cell culture reagents were purchased from Life Technologies (Cergy-Pontoise, France). Other chemicals were from Sigma Chemical Co. (St. Louis, MO). The cDNA of the rat B_2 receptor subcloned in pRC/CMV was kindly provided by Prof. J. Navarro (University of Texas Medical Branch, Galveston, TX). [^{3}H]Bradykinin and [^{3}H][des-Arg^{10},Leu^9]-kallidin (80–120 Ci/mmol; 1 Ci = 37 GBq) were purchased from New England Nuclear.

Cloning of the Human B_2 and B_1 Receptors. CHO cells expressing the human B_2 receptor rat have been previously characterized[40] as well as the HER293 expressing the human B_1 receptor.[39]

Cell Culture and Transfection. CHO cells were maintained in HAM F12 containing 10% fetal calf serum, 4.5 g/L glucose, 100 mg/L streptomycin and 10^5 units/L penicillin. Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum, 4.5 g/L glucose, 1% Glutamax (v/v), 1% nonessential amino acids (v/v), 1 mM sodium pyruvate, 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were transfected with the different cDNA containing vectors (10 μg/plate of 150 mm in diameter) using the calcium phosphate precipitation method. After 48–72 h of recovery, the selection of transfectants was performed using 500 μg/mL Geneticin. Cell clones were isolated by dilution plating, screened for receptor expression and then propagated.

Binding Studies. Stably transfected CHO cells were scrapped from dishes in 5 mL of binding buffer containing 20 mM TES (pH 6.8), 1 mM 1,10-phenantroline, 140 μg/mL bacitracine and 0.1% bovine serum albumin; 293 cells stably transfected with the B_1 receptor were treated as described above except that TES was used at 25 mM and pH 7.4. Cell membranes were obtained by centrifugation (40000*g* for 15 min). Competition binding experiments were carried out by incubating membranes with the competitor ligands and 400 pM [^{3}H]bradykinin for the B_2 receptor (0.5 mL final volume for 90 min) or 1 nM [^{3}H][des-Arg^{10},Leu^9]-kallidin for the B_1 receptor (0.5 mL final volume for 60 min). Nonspecific binding was determined in the presence of 10 μM bradykinin or [des-Arg^{10},Leu^9]-kallidin. Reactions were terminated by filtration with a Brandel cell harvester through Whatman GF/B filters presoaked for 2 h in poly(ethylenimide) 0.1% (w/v). Filters were washed three times with ice-cold 50 mM TES or Tris and the radioactivity retained onto the filters was counted with a Beckman liquid scintillation counter. Protein concentration was measured by the method of Bradford.

Human Umbilical Vein (HUV) Contraction. Human umbilical cords were collected post-delivery and immediately placed in Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.5, and EDTA 0.026. Umbilical vein rings (3–4 mm in length) were incubated overnight for B_1 induction at 37 °C in Krebs solution containing 100 U/mL penicillin and 100 μg/mL streptomycin (to reduce bacterial growth) bubbled with 95% O_2 and 5% CO_2. Tissues were set up in 8-mL jacketed organ baths containing Krebs solution maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2. Strips were left unstretched for 1 h, during which the bath fluid was changed every 15 min with fresh solution. Strips were then stretched to 1 g. All rings were contracted twice by KPSS (Krebs solution in which NaCl was replaced by KCl) in order to obtain the maximal contraction. After the rings were washed twice with normal Krebs solution and returned to the baseline, captopril (10 μM), DL-thiorphan (1 mM), mepyramine (1 μM), atropine (1 μM), indomethacin (3 μM), NG-nitro-L-arginine (30 μM) and nifedipine (0.1 μM) were added into the organ bath. Thirty minutes later concentration–response curves to BK (for activity at B_2 receptors) or [des-Arg^{10}]-kallidin ([des-Arg^{10}]-KD) (for activity at B_1 receptors) were obtained in the presence of vehicle or antagonist added 15 min before. Each ring was used for a single concentration–response curve. The contractile responses to agonists are expressed as percent (%) of the maximal contraction obtained by adding the thromboxane A_2 mimetic, U46619 (1 μM).

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